

Laccase from Sycamore Maple (*Acer pseudoplatanus*) Polymerizes Monolignols

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ABSTRACT

Current understanding of the final oxidative steps leading to lignin deposition in trees and other higher plants is limited with respect to what enzymes are involved, where they are localized, how they are transported, and what factors regulate them. With the use of cell suspension cultures of sycamore maple (*Acer pseudoplatanus*), an in-depth study of laccase, one of the oxidative enzymes possibly responsible for catalyzing the dehydrogenative polymerization of monolignols in the extracellular matrix, was undertaken. The time course for secretion of laccase into suspension culture medium was determined with respect to age and mass of the cells. Laccase was completely separated from peroxidase activity by hydrophobic interaction column chromatography, and its purity was assessed with different types of gel electrophoresis (isoelectric focusing-, native-, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Amino acid and glycosyl analyses of the purified enzyme were compared with those reported from previous studies of plant and fungal laccases. The specific activity of laccase toward several common substrates, including monolignols, was determined. Unlike a laccase purified from the Japanese lacquer tree (*Rhus vernicifera*), laccase from sycamore maple oxidized sinapyl, coniferyl, and *p*-coumaryl alcohols to form water-insoluble polymers (dehydrogenation polymers).

The biosynthesis of lignin was first suggested to proceed from an enzymic dehydrogenation of aromatic precursors by Erdtman (11). In the first *in vitro* demonstration of such an enzyme reaction, coniferyl alcohol was combined with a fungal laccase (*p*-diphenol:O₂ oxidoreductase; EC 1.10.3.1) in the presence of oxygen to produce a DHP¹ with lignin-like characteristics (12). Subsequent studies showed that with the addition of hydrogen peroxide, plant peroxidases (EC 1.11.1.7) could catalyze similar reactions *in vitro* (17). Because peroxidases are broadly distributed among higher plants, whereas laccases have only been detected in a few species, Higuchi (16) proposed peroxidase to be the enzyme primarily responsible for polymerizing the aromatic alcohol precursors (monolignols) of lignin *in vivo*. However, Freudenberg had previously demonstrated that the addition of O₂ to spruce

cambium extracts stimulated DHP formation from coniferyl alcohol more effectively than the addition of H₂O₂, from which he concluded that the combined actions of both laccase and peroxidase are important for lignin biosynthesis in plants (12). Subsequent studies of a purified laccase from the Japanese lacquer tree (*Rhus vernicifera*) found that the enzyme could not oxidize coniferyl alcohol to produce a DHP (26), thus supporting Higuchi's proposal and leading many researchers to believe that laccase plays no role in lignin biosynthesis. Further evidence to support the contention that laccase is not involved in lignin biosynthesis was provided by histochemical studies in which a chromogenic substrate for fungal laccases and peroxidases, syringaldazine, was oxidized when applied to lignifying plant tissues in the presence but not the absence of H₂O₂ (15).

On the other hand, recent studies of plant laccase and peroxidase activities suggest that syringaldazine oxidation may not be an adequate marker for lignin-specific enzyme activity. In contrast to the results of Harkin and Obst (15), Goldberg *et al.* (14) found low levels of syringaldazine oxidation in poplar stem tissue sections even without the addition of H₂O₂; however, these authors attributed this to low levels of endogenous H₂O₂ in the tissues. Additionally, although syringaldazine has been found to be a good substrate for most, if not all, fungal laccases and peroxidases, it is not a substrate for at least one purified plant laccase (18), nor is it a substrate for an extracellular plant peroxidase whose synthesis is correlated with tissue lignification (9). Thus, Nakamura's demonstration that a purified plant laccase could not oxidize coniferyl alcohol (26) remains the primary argument against the involvement of laccase in lignin biosynthesis.

Cell suspension cultures of sycamore maple (*Acer pseudoplatanus*) have been an important model system for the analysis of cell wall carbohydrate structure (25), and they have been reported to synthesize lignin-like compounds under certain conditions (7). *A. pseudoplatanus* cells have also been shown to secrete large quantities of laccase into suspension culture medium (3). As part of an effort to understand the enzymic regulation of lignin biosynthesis in plant cell walls, we have undertaken a study of the substrate specificity and reaction kinetics of this extracellular laccase. Evidence is presented here to show that this plant laccase can oxidize coniferyl alcohol, as well as the other monolignols, resulting in the production of water-insoluble DHPs.

¹ Abbreviations: DHP, dehydrogenation polymer; IEF, isoelectric focusing; ABTS, 2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonate); FPLC, fast protein liquid chromatography; DMF, dimethylformamide; CTAB, cetyltrimethylammonium bromide.

MATERIALS AND METHODS

Chemicals and Reagents

Monolignols were provided by W.H. Morrison, Plant Structure and Composition Research Laboratory (U.S. Department of Agriculture Agricultural Research Service, Athens, GA). Aromatic substrates were obtained from Aldrich Chemical or Sigma. SDS-PAGE mol wt markers were from Bio-Rad, and IEF-PAGE and gel filtration chromatography standards were from Sigma. Phenyl-Sepharose was also from Sigma. Polystyrene standards for HPLC calibration were from Aldrich Chemical and Polysciences, Inc. All other reagents were obtained at the highest purity available and used without further purification.

Cell Suspension Cultures

Sycamore maple (*Acer pseudoplatanus*) cells were cultured in the dark on medium modified by addition of supplemental copper as described by Bligney *et al.* (4). Transfers of cells to fresh medium were performed on a weekly basis, and the absence of fungal and bacterial contamination was verified for each culture.

Time course results represent the average values from two individual flasks (250-mL flasks containing 50 mL of medium) harvested at 2-d intervals. Cells were harvested by filtration on paper, and their wet weights were recorded. Laccase and peroxidase activities in 10- μ L aliquots of filtrate were determined as detailed below.

Protein and Enzyme Assays

Protein was quantitated by the enhanced alkaline copper (Lowry) protein assay (30). Laccase activity was routinely measured spectrophotometrically by following the oxidation of ABTS (6). Suitably diluted enzyme was added to 0.03% ABTS (w/v) in 100 mM sodium acetate, pH 5.0, and the absorbance at 420 nm was measured over time with a Varian DMS200 scanning spectrophotometer. Spectral scans were taken from 230 to 700 nm. The extinction coefficient of oxidized ABTS was taken as $\epsilon_{420} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Peroxidase activity was routinely assayed spectrophotometrically by following the oxidation of guaiacol (24). Appropriately diluted enzyme was added to a reaction mixture containing 13 mM guaiacol and 5 mM H_2O_2 in 40 mM Tris-HCl, pH 6.8. The increase in absorbance at 436 nm was measured over time, and the extinction coefficient for oxidized guaiacol was taken as $\epsilon_{436} = 6.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Enzyme Purification

Spent medium from 12-d-old cultures of sycamore maple cells was recovered by filtration through sintered glass. All subsequent purification steps were performed at 4°C. The crude filtrate (4.0 L) was immediately concentrated to 700 mL by tangential-flow ultrafiltration against a membrane cassette with a 10-kD molecular mass cutoff (Pellicon; Millipore), followed by concentration to 200 mL with the use of stirred cell ultrafiltration against a PM-10 membrane (Ami-

con). The retentate was dialyzed extensively against 2.0 M potassium phosphate, pH 6.7, centrifuged to remove insoluble matter (recovery of 75 mL) and applied to a phenyl-Sepharose column (2.6 \times 30 cm) equilibrated in the same buffer. With gravity flow, the column was washed with 200 mL of loading buffer, and enzyme was eluted with a 1.0-L linear gradient of 2.0 to 0.02 M potassium phosphate, pH 6.7, followed by a linear gradient of 0 to 15% ethylene glycol in 20 mM potassium phosphate, pH 6.7. Fractions of about 13.0 mL were collected and eluted protein was monitored by absorbance at 280 nm. Fractions containing laccase activity were pooled and concentrated to 12.5 mL via stirred cell ultrafiltration. After a final concentration to 2.3 mL was attained by centrifugal ultrafiltration (Centricon 10, Amicon), the purified laccase was stored frozen at -70°C.

Gel Electrophoresis and Molecular Mass Determination

The mol wt of native laccase was estimated by gel filtration FPLC on a Superose 12 (Pharmacia) column. The column was calibrated with β -amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), horseradish peroxidase (40 kD), and carbonic anhydrase (29 kD) as molecular mass markers. Samples (200 μ L) were applied to the column in 50 mM sodium acetate buffer, pH 5.0, containing 150 mM NaCl and eluted at a flow rate of 0.3 mL/min.

PAGE was run under denaturing (SDS-PAGE) or non-denaturing (native-PAGE) conditions according to the procedures of Laemmli (20). Proteins were visualized by silver staining (5), and the molecular mass of laccase was estimated by comparison with rabbit phosphorylase b (97.4 kD), BSA (66.2 kD), ovalbumin (45.0 kD), carbonic anhydrase (31.0 kD), bovine trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD) molecular mass markers. The isoelectric point of laccase was determined in comparison to IEF standards by running pH 3.0 to 9.0 and 4.0 to 6.5 gels in a PHAST system (Pharmacia-LKB). Proteins in PHAST gels were detected with the use of the Pharmacia silver stain kit according to the manufacturer's instructions. For laccase and peroxidase activity staining, gels were incubated in ABTS and guaiacol reaction mixtures, respectively, as described under "Protein and Enzyme Assays."

Amino Acid and Glycosyl Analyses

Amino acid composition determinations and N-terminal protein sequence analysis were performed at the Molecular Genetics Facility, University of Georgia. Amino acid composition results represent the average for two separate hydrolysis and derivatization runs. Neutral sugar concentrations (from 40 μ g of protein) were estimated by the anthrone method of Dische (10). Determinations of glycosyl composition were performed by GC and GC-MS analysis of the acetylated alditol derivatives obtained after hydrolysis (2.0 M TFA, 120°C, 2 h), reduction (1% NaBD_4 [w/v] in alkaline ethanol), and acetylation (1:1 pyridine:acetic anhydride, 120°C, 20 min) of the protein (21).

Substrate Specificity and Reaction Rates

Unless noted otherwise, substrate specificities were determined in 20 mM potassium phosphate buffer, pH 6.8, at selected substrate concentrations. For reactions in which the substrate and product had overlapping absorbance spectra at the extinction maximum (*p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and hydroquinone), the reaction rate was calculated assuming that the absorbance would fall to zero if all of the substrate were consumed. The remaining reaction rates were determined from the increased absorbance due solely to the product at the extinction maximum. Enzyme activity, estimated from the initial rate of reaction, was expressed as the number of micromoles of substrate oxidized per min at 30°C for the defined conditions. Spectrophotometric determinations of monolignol oxidation were made by mixing laccase (56 µg) with *p*-coumaryl (33.3 µM), coniferyl (55 µM), or sinapyl alcohol (148 µM) in 20 mM K-phosphate buffer, pH 6.8, in a 1-mL spectrophotometric cell at 22°C. The absorbance of each reaction mixture was scanned between 230 and 400 nm at 2.5-min intervals, except in the case of *p*-coumaryl alcohol, for which two scans were made 10 h apart. Effectors of diphenoloxidases—cinnamic, *p*-coumaric, and ferulic acids, CTAB, and SDS (34)—or tyrosinases—tropolone (19)—were tested for inhibitory or activating activities against *A. pseudoplatanus* laccase by addition to a reaction mixture composed of 20 mM sodium phosphate buffer, pH 6.8, containing 8 mM pyrogallol.

DHP Analysis

Polymerization of monolignols was performed in 20 mM sodium phosphate buffer, pH 6.8, containing 10 mM *p*-coumaryl, coniferyl, or sinapyl alcohol. Laccase (approximately 5 µg) was added to a 1-mL reaction, which was subsequently incubated for 24 h at 26°C on an orbital shaker. The water-insoluble DHPs were recovered by centrifugation. Before analysis by gel-permeation HPLC, DHP pellets were dissolved in DMF and aliquots were diluted to an approximate concentration of 10 µg/mL. The DHPs (20 µL) were separated by HPLC on a column (8 × 300 mm) packed with a styrene-divinylbenzene resin (Shodex KD-802, Millipore). The column was eluted with DMF at 55°C and a flow rate of 1 mL/min. DHPs were detected by absorption at 280 nm and the elution profiles were compared to those for the pure monolignols. The void volume of the column in this system was 5.0 mL, as determined with the use of polystyrene standards (24 and 48 kD).

RESULTS

Laccase activity in sycamore maple cell suspension medium reached a maximum approximately 12 d after inoculation, just before the cells entered stationary phase (Fig. 1). Extracellular peroxidase activity continued to increase after this point, as did cell death and lysis. To minimize contamination of the culture medium with intracellular enzymes released from lysing cells, cultures were routinely harvested on the 12th d of culture.

The laccase from spent *A. pseudoplatanus* medium was purified by a combination of ultrafiltration and hydrophobic

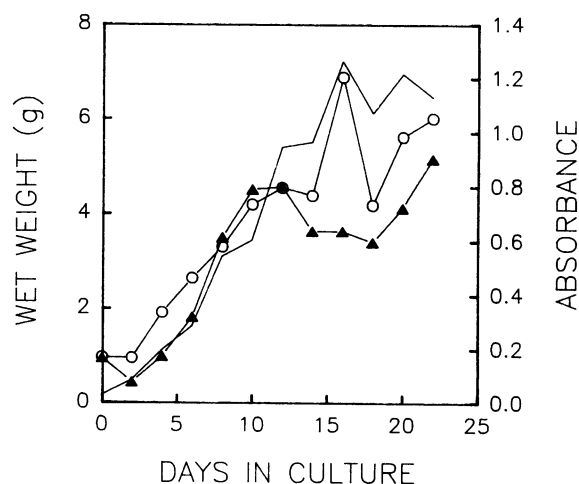


Figure 1. Time course of laccase and peroxidase production by sycamore maple cells grown in suspension culture. The average wet weight of cells, as well as laccase and peroxidase activity from two separate flasks, were determined after filtration. The solid line represents cell wet weight (g), the ▲ represent laccase activity (A_{420}), and the ○ represent peroxidase activity (A_{436}).

interaction column chromatography, as described in "Materials and Methods." Three peaks of laccase activity eluted from the phenyl-Sepharose column (Fig. 2), but results presented here were obtained using only the enzyme contained in the earliest eluting peak of laccase activity (fractions 53–68). Because the oxidation rate for coniferyl alcohol by the enzyme in this pool actually declined slightly upon addition of H_2O_2 , this laccase preparation was considered free of any peroxidase activity. Concentration of the laccase pool (fractions 53–68) by ultrafiltration (Centricon 10, Amicon) yielded a solution containing 4.4 mg of protein (1.5 mg/mL) with the deep-blue color indicative of laccase. A spectrophotometric scan of this preparation demonstrated absorption bands at 278 nm (with a shoulder at 290 nm), corresponding to aromatic amino acids, a second shoulder between 330 and 340 nm, and a peak at 612 nm. According to Reinhammar and Oda (28), absorption bands at 330 to 340 and 612 nm correspond to Type 3 and Type 1 Cu(II), respectively.

A native laccase molecular mass estimate of 115 ± 15 kD from gel filtration chromatography was essentially the same as the value obtained by Bligny and Douce (3) with the use of ultracentrifugation. SDS-PAGE analysis of the purified laccase demonstrated a single polypeptide having a mol wt of approximately 97,400 (Fig. 3A). The diffuse nature of the polypeptide band found by SDS-PAGE was identical to that found by Bligny and Douce (4), and probably reflects the heavy glycosylation of sycamore maple laccase (31). Based on the mol wt, as determined by SDS-PAGE, protein quantity as measured in the alkaline copper assay, and spectrophotometric analysis, the ϵ_{612nm} and ϵ_{280nm} for *A. pseudoplatanus* laccase may be estimated as $2.4 \times 10^3 M^{-1} cm^{-1}$ and $10.5 \times 10^4 M^{-1} cm^{-1}$, respectively. The latter value may be compared to that for a laccase purified from *Neurospora crassa*, reported as $9.07 \times 10^4 M^{-1} cm^{-1}$ (13).

As was the case with SDS-PAGE, native-PAGE (Fig. 3B)

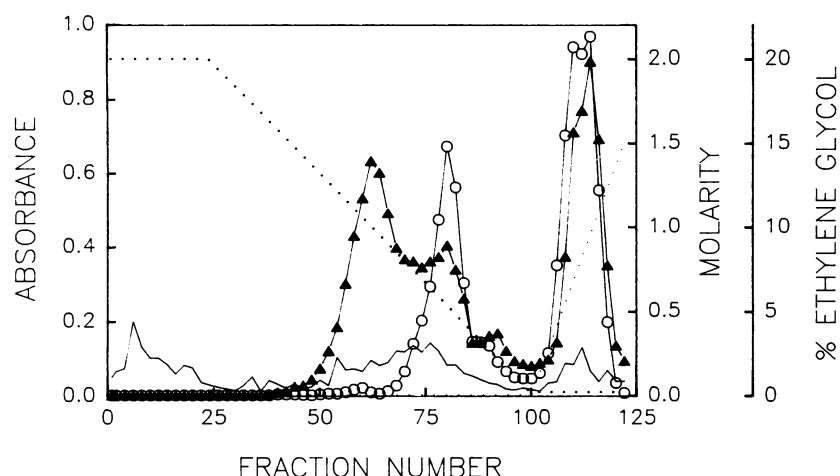


Figure 2. Separation of *A. pseudoplatanus* laccase from peroxidases by phenyl-Sepharose column chromatography. Column condition were as described in "Materials and Methods." Protein (A_{280}) is represented by the solid line, the \blacktriangle represent laccase activity (A_{420}), and the \bigcirc represent peroxidase activity (A_{436}). The dotted lines represent the descending salt gradient (large dots) and ascending ethylene glycol gradient (small dots).

and IEF-PAGE (pH 3–9) (not shown) analysis demonstrated single, but diffuse, bands by activity staining, as well as by silver staining. However, IEF of purified laccase from pH 4.0 to 6.5 revealed several discrete bands harboring laccase activity. From the position of IEF marker proteins in PHAST system IEF-PAGE gels, these bands appeared to focus between pH 4.0 and 5.2, with the predominant bands focusing around pH 5.2. No peroxidase activity was detected in any of these gels.

Amino terminal sequence analysis of the purified laccase found only a single polypeptide sequence—Ala-Ile-Arg-His-Tyr-Asp-Phe-Val-Val-Lys-Glu-Ser—suggesting that the multiple bands detected on narrow range IEF gels are caused by microheterogeneities in the glycosyl substituents (2). Amino acid analysis of *A. pseudoplatanus* laccase indicated that the protein contained a relatively high number of aspartate/asparagine residues (Table I). This likely reflects the involve-

ment of asparagine residues in the numerous glycosyl side chains identified in this protein by other researchers (31). A total carbohydrate determination of approximately 40% for laccase compares favorably with the estimate of these same authors. By using 40% as the total carbohydrate content of laccase and calculating from the molecular mass value determined by gel filtration chromatography (115 ± 15 kD), the polypeptide backbone of sycamore maple laccase should have a molecular mass of 69 ± 9 kD. Glycosyl composition analysis for the purified laccase showed the presence of glucose (19%), arabinose (6%), galactose (21%), fucose (3%), xylose (3%), mannose (22%), and *N*-acetylglucosamine (23%).

As is the case with other laccases, *A. pseudoplatanus* laccase

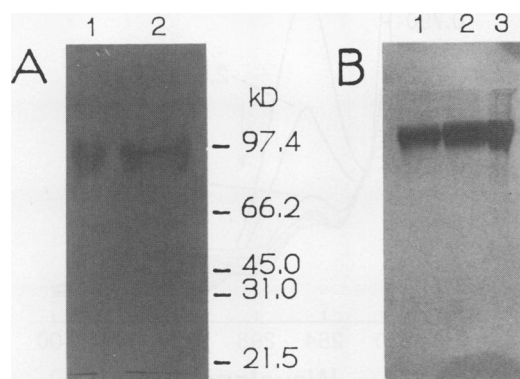


Figure 3. Analysis of purified *A. pseudoplatanus* laccase by gel electrophoresis. A, SDS-PAGE analysis: lane 1, 8 μ g of laccase; lane 2, 7 μ g of laccase. Polypeptides were visualized by silver staining. B, Native-PAGE analysis: lane 1, 4 μ g of laccase; lane 2, 8 μ g of laccase; lane 3, 11 μ g of laccase. Polypeptides were visualized by silver staining. The same bands in other lanes were from the same gel containing identical amounts of enzyme stained positive for laccase activity with ABTS (not shown). Molecular mass values correspond to polypeptide standards run under denaturing conditions in panel A.

Table I. Comparison of *A. pseudoplatanus* Laccase Amino Acid Composition with Those of Laccases from a Fungus, *Neurospora crassa* (13), and a Plant, *Rhus vernicifera* (27).

Amino Acid ^a	No. of Residues/mol Sycamore Laccase ^b	No. of Residues/mol <i>N. crassa</i> Laccase ^c	No. of Residues/mol <i>R. vernicifera</i> Laccase ^d
Alanine	42.9	35.0	39.0
Arginine	18.8	21.0	12.5
Aspartate + asparagine	114.0	77.0	60.0
Glutamate + glutamine	58.4	35.0	36.6
Glycine	51.0	48.0	36.0
Histidine	21.4	16.0	16.8
Isoleucine	25.4	27.0	31.1
Leucine	28.0	38.0	34.3
Lysine	20.9	20.0	26.0
Methionine	4.5	6.0	10.7
Phenylalanine	25.0	19.0	30.2
Proline	47.6	33.0	37.5
Serine	40.0	27.0	37.0
Threonine	62.3	34.0	49.7
Tyrosine	17.7	20.0	24.4
Valine	32.0	32.0	43.0

^a Cysteine and tryptophan were not determined. ^b Molecular mass = 66 kD. ^c Molecular mass = 64.8 kD. ^d Molecular mass = 110 kD (estimated from carbohydrate content).

Table II. Specific Activity of *A. pseudoplatanus* Laccase with Various Substrates

Substrate	Molarity	Wavelength	ϵ_{\max}	Specific Activity
	mM	nm	M ⁻¹ cm ⁻¹	$\mu\text{mol min}^{-1}$ mg ⁻¹
<i>p</i> -Coumaryl alcohol ^a	0.08	259	13,210	0.032
Coniferyl alcohol ^a	0.11	264	13,090	0.40
Sinapyl alcohol ^a	0.21	272	8,093	1.1
Hydroquinone ^a	5.5	250	17,254	5.9
Phloroglucinol	1.9	268	ND ^f	0
Phenol	0.17	270	ND ^f	0
Catechol ^b	20	450	2,211	10.6
4-Methylcatechol ^b	20	420	2,091	7.8
Guaiacol ^{b,c}	13	436	6,400	0.53
Hydroxyquinol ^{b,d}	4.7	490	2,857	102.1
Pyrogallol ^b	20	450	4,400	7.4
Syringaldazine ^b	0.1	550	27,076	0.93
ABTS ^{b,e}	2.3	420	36,000	7.9

^a Reaction rate determined from the decrease in absorbance at the extinction maximum. ^b Reaction rate determined from the increase in absorbance at the extinction maximum. ^c Assayed in 40 mM Tris-HCl, pH. 6.8. ^d Assayed in H₂O. ^e Assayed in 100 mM sodium acetate, 6.8 pH 5.0. ^f ND, Not determined.

demonstrated a broad substrate specificity. In addition to oxidizing all three monolignols, the enzyme readily oxidized several chromogenic substrates, including syringaldazine (Table II). Besides its capacity to oxidize hydroquinone, the ability of the purified enzyme to oxidize pyrogallol in the presence of cinnamic acids, tropolone, and CTAB (Table III) demonstrated it to be a laccase-type, *p*-diphenol:O₂ oxidoreductase rather than a tyrosinase or *o*-diphenoloxidase (EC 1.10.3.2) (19, 34). Timed spectrophotometric scans taken of reaction mixtures containing sycamore maple laccase and *p*-coumaryl, coniferyl, or sinapyl alcohol are depicted in Figure 4. Laccase oxidized sinapyl alcohol more rapidly than coniferyl alcohol, but both of these reactions were complete within minutes. On the other hand, *p*-coumaryl alcohol was oxidized very slowly, taking several hours for the reaction to reach completion. During oxidation of sinapyl alcohol, absorbance at 332 nm increased with time, suggesting the formation of a quinone-like side product (Fig. 4C).

Addition of purified laccase to more concentrated solutions of the monolignols led to the production of opaque suspensions characteristic of DHP formation. DHP formation still occurred even when catalase was added to the reaction

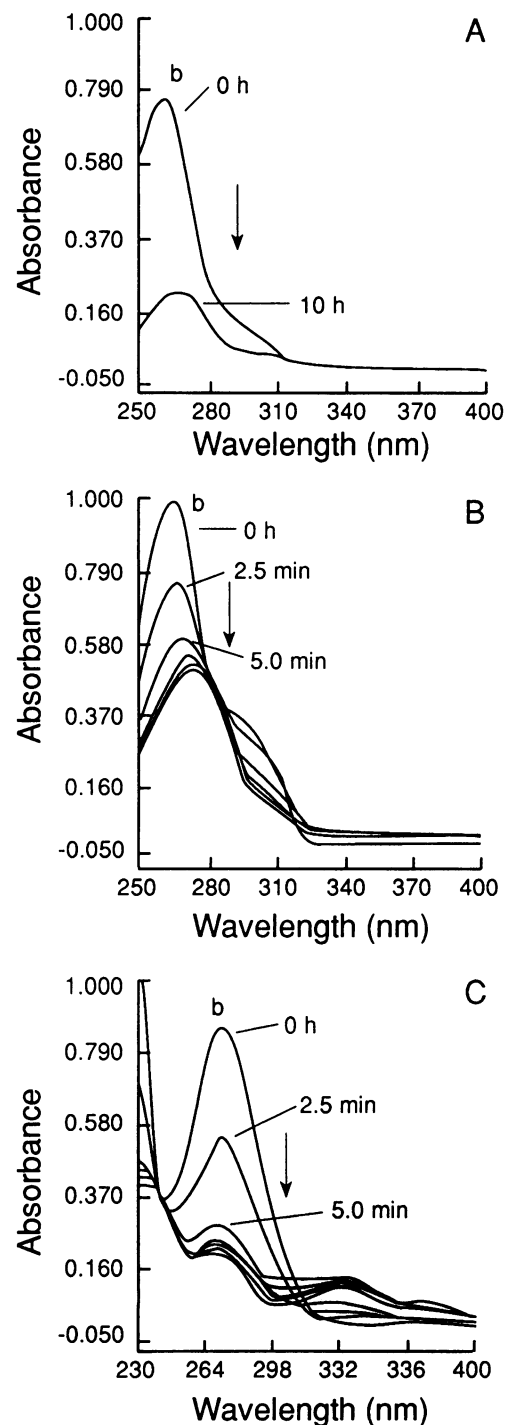


Figure 4. Monolignol oxidation by laccase purified from *A. pseudoplatanus*. Sycamore maple laccase (56 μg) was incubated at 22°C in a thermostatted cuvette with *p*-coumaryl (A), coniferyl (B), or sinapyl alcohol (C). Spectrophotometric scans were taken every 2.5 min for the oxidation of coniferyl and sinapyl alcohols. The two scans to illustrate *p*-coumaryl alcohol oxidation were taken 10 h apart. Arrows indicate the decline in absorbance that accompanied monolignol oxidation catalyzed by laccase when compared to absorbance curves in blanks containing only the enzyme (b).

Table III. Effect of Various Phenoloxidase Inhibitors on the Activity of *A. pseudoplatanus* Laccase

Substrate	Molarity	Inhibition
	mM	%
Cinnamic acid	16	0
<i>p</i> -Coumaric acid	15	0
Ferulic acid	1.2	0
Tropolone	0.5	0
CTAB	11	0
SDS	1.0	20

mixtures in order to completely remove any residual H_2O_2 . The percent yields (w/w) for production of water-insoluble DHPs by the purified laccase were 33 ± 4 , 56 ± 4 , and $14 \pm 1\%$ for *p*-coumaryl, coniferyl, and sinapyl alcohol, respectively. The speed with which DHPs were formed paralleled the spectrophotometric measurements of monolignol oxidation. Gel permeation HPLC and UV absorbance analysis of the reaction mixture precipitates showed that the water-insoluble material was an aromatic polymer (Fig. 5). Because of difficulties imposed by the tendency of aromatic polymers to aggregate (8, 23), it is not possible to draw firm conclusions from gel permeation chromatography as to the exact mol wts of the DHPs produced with sycamore maple laccase. However, a comparison of the three elution profiles shows that precipitates from the reactions with *p*-coumaryl alcohol contain a higher proportion of high mol wt polymer, whereas precipitates from the sinapyl alcohol reaction contain large amounts of material that coelutes with monomeric sinapyl alcohol. More detailed characterizations of the materials in the eluted peaks are currently underway.

DISCUSSION

In our hands, a purification protocol developed for *A. pseudoplatanus* laccase that uses Con A affinity chromatography (3) did not yield an enzyme completely free of peroxidase activity. For subsequent work, separation of these activities was particularly important given the observation that some peroxidase isozymes appear to harbor other phenoloxidase (O_2 oxidoreductase) activities (1). Separation of laccase into three activity peaks by hydrophobic interaction chromatography was unexpected (Fig. 2), and the basis for this

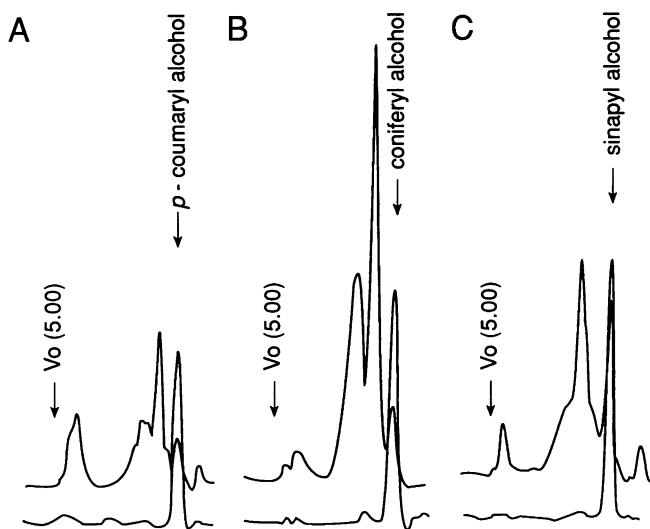


Figure 5. Gel permeation HPLC analysis of DHPs produced by the action of *A. pseudoplatanus* laccase on each monolignol. DHPs produced from *p*-coumaryl (A), coniferyl (B), or sinapyl (C) alcohols by the action of laccase were fractionated by gel permeation HPLC. Upper traces depict the elution profiles of reaction products monitored by A_{280} , whereas lower traces show the elution peaks of unreacted monolignol substrates (arrows). The void volume (V_0), as determined by the elution of high mol wt polystyrene standards, is indicated by the arrow at 5.00 mL.

result is being investigated. However, the physical parameters, *i.e.* mol wt, absorbance spectrum, and sugar composition, of the protein we have purified appear identical to those reported for the enzyme purified by Bligny and Douce (3).

Laccase purified from the extracellular milieu of suspension-grown sycamore maple cells readily oxidizes coniferyl and sinapyl alcohols to form water-insoluble DHPs (Fig. 5). The enzyme also polymerizes *p*-coumaryl alcohol, but at a much slower rate (Fig. 4). These results challenge conclusions drawn from studies of *Rhus* laccase activity (26) and clearly suggest that laccase may indeed be capable of contributing to lignin biosynthesis. Unlike the laccase purified from mango (18), laccase from *A. pseudoplatanus* can oxidize syringaldazine. Thus, the activity of a laccase enzyme similar to the one we have purified could have been responsible for the syringaldazine oxidation observed by Goldberg *et al.* (14) in the absence of added H_2O_2 . Immunolocalization experiments are underway to verify that laccase is secreted into lignified regions of sycamore maple cell walls *in vivo*.

Sycamore maple cells do not become lignified under the growth conditions used for this study (4); consequently, we cannot yet draw any conclusions as to the relationship between laccase and lignin biosynthesis *in vivo*. Studies of the enzyme and lignin-like materials produced by *A. pseudoplatanus* cells grown under the conditions described by Carcellar *et al.* (7) are underway.

Studies by Terashima and coworkers (32) have elegantly demonstrated the heterogeneity of lignin at the subcellular level, as well as at the tissue and organismal levels. Their studies indirectly suggest that the extracellular oxidative enzymes, or at least the substrate specificities of those enzymes responsible for monolignol polymerization, may change with respect to which monolignols are incorporated into lignin at particular stages of development. Work with peroxidases isolated from several tree species (29, 33) suggests that differences in substrate specificity between particular isozymes could contribute to the developmental differences seen in lignin deposition. If laccase does contribute to lignin biosynthesis *in vivo*, its preference for sinapyl and coniferyl alcohols could likewise contribute to variations in lignin structure. Different isozymes of the extracellular oxidative enzymes might also be responsible for the varied polymer bonding patterns observed between "native" lignins and DHPs synthesized *in vitro* with the use of horseradish peroxidase (22, 23). Thus, a significant factor contributing to lignin heterogeneity in plant cell walls may be the use of a variety of phenoloxidases, *i.e.* peroxidases and laccases, to polymerize lignin under different conditions.

NOTE ADDED IN PROOF

Immunolocalization studies of sycamore maple laccase by Driouch *et al.* (Characterization and localization of laccase forms in stem and cell cultures of sycamore. [1992] Plant J 2:13–24) show the protein to only be in the cell walls of xylem and epidermal cells in the intact plant.

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